Transactions of the Royal Society of S. Aust. (2001), **125**(1), 57-63

EMBRYOGENESIS, CULTURE AND DESCRIPTION OF THE FREE-LIVING STAGES OF TWO NEMATODE PARASITES OF THE NORTHERN HAIRY-NOSED WOMBAT (*LASIORHINUS KREFFTII*) (VOMBATIDAE; MARSUPIALIA)

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Summary

Smales, L. R., Gerhardt, K. & Heinrich, B. (2001) Embryogenesis, culture and description of the free-living stages of two nematode parasites of the northern hairy-nosed wombat (*Lasiorhinus krefftii*) (Vombatidae: Marsupialia). *Trans. R. Soc. S. Aust.* **125** (1), 57-63, 31 May, 2001.

Faecal pellets were collected from the only extant population of *Lasiorhinus krefftii*, the northern hairy-nosed wombat, at Epping Forest National Park, Central Queensland. Nematode eggs and larvae, extracted from these pellets, representing 24 h samples from the total host population, were cultured in the laboratory. The eggs, all presumed to be *Oesophagostomoides eppingensis* hatched as first-stage larvae after 19 – 23½ h in distilled water at 25° C. The optimum temperature for larval hatch in faecal culture was 26° C. All larvae had moulted to second-stage by day 3 and to third stage sheathed larvae by day 5. Third-stage *Strongyloides* sp. larvae, smaller than larvae of *S. spearei* occurring in *Vombatus ursinus*, the common wombat, were also found in the cultures. The developmental strategies of *O. eppingensis* free-living stages, optimum temperature for hatching and unprotected first and second-stage larvae, are congruent with those of strongylid species native to temperate regions outside Australia but not those of the Australian strongyles *Hypodontus macropi*, *Rugopharynx rosmariae*, *Labiostrongylus eugenii* and *Cloacina similis*, all of which have protected second-stage larvae and in the case of the latter two species protected first-stage larvae also.

Key Words: *Lasiorhinus krefftii*, wombat, *Oesophagostomoides eppingensis*, *Strongyloides* sp., nematode life-cycle, free-living stages.

Introduction

Lasiorhinus krefftii (Owen, 1872) (Vombatidae: Marsupialia) the northern hairy-nosed wombat is now restricted to a single population of about 65 individuals in Epping Forest National Park (EFNP) 120 km north west of Clermont (22° 19' S, 146° 47' E), Central Queensland (Crossman *et al.* 1994). Although this species was previously distributed through Queensland, New South Wales and across the border into Victoria, the present reduction in range and numbers is thought to have occurred over the past 120 years (Taylor *et al.* 1994). The northern hairy-nosed wombat is listed as critically endangered (Maxwell *et al.* 1996) and a Recovery Plan is being managed through the Environment Protection Agency of Queensland (Horsup 1999[1]).

The wombats occupy a single burrow system, a series of large burrows arranged in loose clusters along the banks of a sandy gully (Johnson & Crossman 1991). These animals spend 2 - 6 h, only at night, above ground. Burrow entrances are marked with piles of fresh faecal pellets with smaller piles deposited along the paths between the burrows but not elsewhere (Johnson & Crossman 1991). Fresh, that is still moist, pellets collected from the burrow system at dawn can therefore provide a 24 h sample from the total extant population of the wombat.

Two species of gastro-intestinal nematode *Oesophagostomoides eppingensis* Smales, 1994 (Strongylida: Strongylidae) and *Strongyloides* sp. (Rhabditida: Strongyloididae) have been found in the northern hairy-nosed wombat (Smales 1998; Gerhardt *et al.* 2000). Of these two species only one, *O. eppingensis*, produces eggs that pass out with the faeces. Any eggs extracted from wombat faeces collected from EPNS are therefore probably eggs of *O. eppingensis*

During 1996 and again in 1999 the opportunity arose to collect fresh faecal samples from the wombat population in EFNP. This activity was sanctioned by the Recovery Team. From these samples we were able both to isolate nematode eggs and culture larval nematodes. This enabled us to investigate the embryogenesis and hatching of eggs of *O. eppingensis* and to determine the morphology of *O. eppingensis* and *Strongyloides* sp. larvae.

Materials and Methods

Fresh faecal pellets, collected from EFNP in June and August 1996, and August and September 1999 were transported to Rockhampton on ice and stored at 4° C. Eggs for embryological study were extracted from small amounts of faecal material that had been sedimented with distilled water. Fifteen eggs

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[1] Horsup, A. (1999) Recovery plan for the northern hairy-nosed wombat (*Lasiorhinus krefftii*) 1998 – 2002. Report submitted to Environment Australia by the Northern Hairy-nosed Wombat Recovery Team through the Department of Environment and Heritage, Queensland (unpub.).

were measured, placed in hanging drops, held at room temperature (25° C), observed hourly using an Olympus CH3 differential interference microscope, and photographed.

The number of eggs per gram of faeces in the pellets was determined by the standard McMaster technique[2]. Faecal cultures were then established using 1 g faeces, 1 g activated charcoal (8 mm diameter), 5 ml water and 5 drops 4% Nystatin, placed on filter paper in Petri dishes. Preliminary trials were carried out in 1996 and cultures were set up at 18°, 22°, 26° and 28° C for seven days in 1999. The larvae collected were examined live in water or after killing in hot 70% ethanol and clearing in a mixture of 70% ethanol and glycerine and being left until the alcohol had evaporated. Larvae were differentiated into strongyloidid and strongylid forms and the total number of strongylids hatching after seven days, at each temperature, was recorded.

Six strongylid larvae were measured on hatching from the hanging drop preparations described above. First-stage strongylid larvae, collected from a faecal culture one day after its establishment, were transferred into a Petri dish and maintained in a incubator at 28° C for four days. Ten larvae were recovered and measured on establishment of the cultures and a further 10 larvae were removed and measured each day for the next three days.

Results

By the time the eggs had been transported from EFNP, extracted from faecal pellets and established in hanging drops, embryological development had already commenced, with the embryos having reached about a 16 cell stage. Eggs measured 79 – 92 (88) µm by 42 – 51 (46) µm. The stages of development are shown in Fig. 1 and a time chart of the sequence is given in Table 1.

Elongation of the embryo began at about 9 h, the larviform embryo began actively moving after 10 h, the oesophagus was clearly visible after about 16½ h and the intestine could be differentiated from 18 h. Hatching as first-stage larvae occurred after 19 – 23½ h. Hatching involved the larva twisting actively in a continuous figure-of-eight pattern followed by a pulsating movement against the side of the egg. This pulsating was associated with a bulge in the eggshell followed by the shell rupturing and the larva emerging as a first-stage larva head or tail first. The hatching process took 2 – 10 min.

Results from the preliminary trials indicated that hatching occurred successfully between 18° and 30° C but not at 4° C. The cumulative numbers of nematodes that had hatched after four days in faecal culture are given, as the percentage hatch, in Fig. 2, and were compared among the four temperature treatments using a Chi squared 2 x 4 contingency table analysis of the proportion of hatched versus unhatched. There was a significant difference among treatments ($\chi^2_3 = 47.49$, $P < 0.001$), so an iterative *a posteriori* analysis was done by progressive removal of the most extreme treatment group. The results of this analysis showed there was no difference in the proportion hatching at 18 or 22° C ($\chi^2_1 = 0.27$, N.S.), but the proportion hatching differed significantly among temperatures of 18, 22 and 28° C (with a smaller proportion hatching at 28° C: $\chi^2_1 = 11.72$, $P < 0.01$) and also among temperatures 18, 22, and 26° C (with a larger proportion hatching at 26° C: $\chi^2_1 = 20.36$, $P < 0.001$). Finally, there was a significant difference between 26 and 28° C in the proportion hatching ($\chi^2_1 = 12.31$, $P < 0.001$). Taken together, these tests differentiated three statistically significant groups. Hatching was lowest at 28° C, intermediate at 18 and 22° C, and higher than both of these groups at 26° C.

Although during collection, transport and storage

TABLE 1. *Time chart of developmental sequence of* Oesophagostomoides eppingensis *eggs kept in hanging drops of distilled water at 25° C.*

Stage	Time (h)	3	6	9	12	15	18	21	24
morula		———							
elongation begins			———						
vermiform embryo				———					
mouth visible					———	———	———		
oesophagus visible						———	———		
embryo 3 x length of egg					———	———	———		
intestine well defined							———		
hatching							———		

School of Veterinary Science (1998) Laboratory Manual for Veterinary Parasitology. University of Melbourne (unpub.).

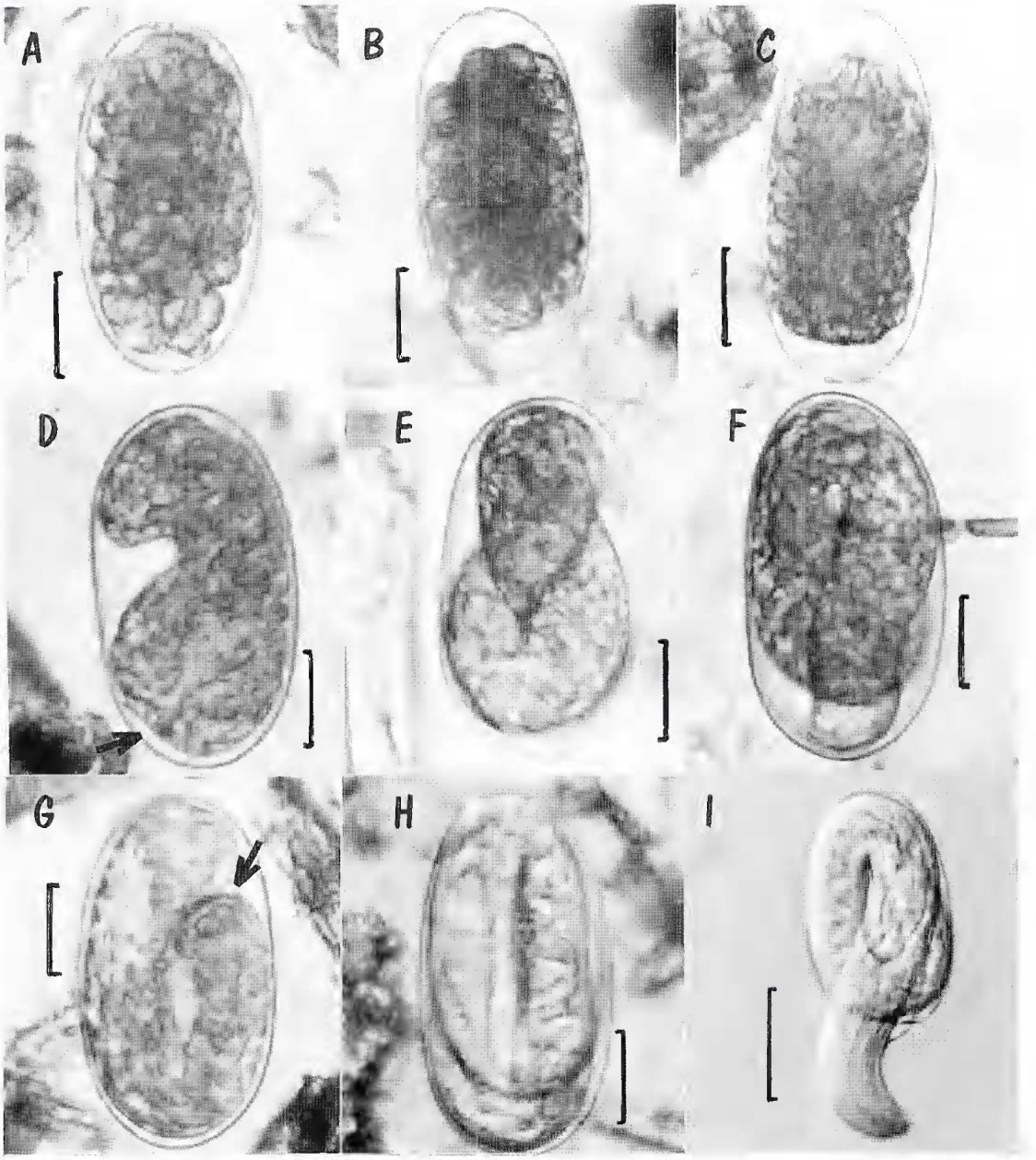

Fig. 1. *Oesophagostomoides eppingensis* eggs kept in hanging drops in distilled water at 25° C. A. After 4 hours. B. After 5 hours. C. After 7 hours, elongation beginning. D. After 9 hours. E. After 11 hours, larviform embryo, elongated to twice the length of the egg, now active. F. After 13 hours, oesophagus forming. G. After 15 hours, larva about three times length of egg. H. After 18 hours, intestine visible. I. After 20 hours, beginning to hatch. Arrows indicate developing mouth. Scale bars = 20 µm.

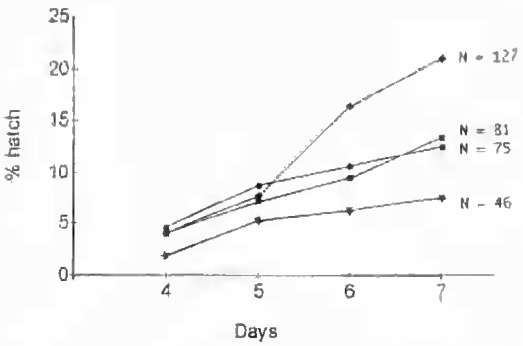

Fig. 2. Percentage of *Oesophagostomoides eppingensis* eggs harvested as larvae from faecal cultures held at a range of temperatures for 7 days —•—•— = 18° C. —■—■— = 22° C. —◆—◆— = 26° C. —+—+— = 28° C. N= the number of eggs hatched for each treatment.

faecal pellets were kept moist at about 4° C, the time in storage may have affected the viability of the eggs and hence the overall relatively low hatching rate at all temperatures. Further, the logistics of the exercise precluded both the collection of large amounts of material at any one time and multiple collection events. Notwithstanding these limitations, an informative set of data has been collected.

Measurements of the strongylid larvae are given in Table 2. All larvae had moulted to second-stage by day 3 and to sheathed third-stage by day 5 (Figs 3 & 4). Before moulting the larvae attached themselves to the substrate by the anterior end. Starting from the posterior end, the cuticular sheath became loose and the larva wriggled backwards until it was free, leaving the sheath firmly attached to the substrate. These larval sheaths were detected in the culture on day 3. Although moults to third-stage were not observed, some cuticularisation of the buccal region and greater definition of the lips was apparent by day 5. A small genital anlage could be seen in the third-stage larvae.

Also detected in all faecal cultures on day one was a number of larvae with rhabditiform oesophagus morphology and by day four, forms with a long filariform oesophagus and a notched tail, typical of infective larvae of species of the genus *Strongyloides*, were also apparent. These larvae were 425 – 470 µm long with the oesophagus 200 – 230 µm long, the tail 40 - 58 µm long and the genital anlage 240 – 300 µm from the anterior end (Fig. 5).

Discussion

The Australian strongylids studied thus far, have free-living larval stages that retain the cuticle of previous moults to sheath subsequent stages. *Rugopharynx vosmariae* Beveridge & Presidente, 1978 and *Hypodontus macropi* Monnig, 1929 both hatch as first stage larvae and retain the sheaths of the first and second moults, so that the second-stage larva is sheathed and the third-stage larva has a double sheath (Beveridge & Presidente 1978; Beveridge 1979). *Labiostrongylus eugenii* Johnston & Mawson, 1940 hatches as a second-stage sheathed larva and moults to a third-stage double sheathed larva (Smales 1977) and *Cloacina similis* Johnston & Mawson, 1939 hatches as a third-stage double-sheathed larva (Clark 1971).

By contrast *O. eppingensis* hatches as a first-stage unsheathed larva and retains only the second-stage cuticle as a single sheath around the third-stage larva. These differences in stage of hatching are reflected in the time taken from the beginning of embryogenesis to hatching, 19 – 23 h for *O. eppingensis*, 12 h for *H. macropi*, 20 – 40 h for *R. vosmariae* and 67 – 114 h for *L. eugenii* (Smales 1977; Beveridge & Presidente 1978; Beveridge 1979). The time *O. eppingensis* took to hatch and then develop to third-stage sheathed larva (4 days at 28° C) is consistent with the life cycle patterns given by Anderson

TABLE 2. *Measurements (µm) of* Oesophagostomoides eppingensis *in distilled water culture at 25° C.* Ten larvae were removed and mensured each day. The range is followed by the mean.

Day	Larval Stage	Length	Width	Oesophagus length
0	1	277 – 323 (292)	22 – 27 (24)	58 – 77 (69)
1	1	325 – 365 (344)	19 – 25 (22)	73 – 83 (78)
2	1	355 – 383 (369)	21 – 27 (24)	88 – 98 (93)
3	2	384 – 416 (400)	22 – 28 (25)	93 – 103 (98)
4	2	438 – 462 (450)	26 – 30 (28)	95 – 111 (103)
5	3	490 – 545 (528)	28 – 41 (34)	119 – 132 (128)

CLARK, H. (1971) A preliminary investigation into the free-living stages of two nematode parasites of the Kangaroo Island Wallaby (*Macropus eugenii*). BSc. (Hons) Thesis, The University of Adelaide (unpub.).

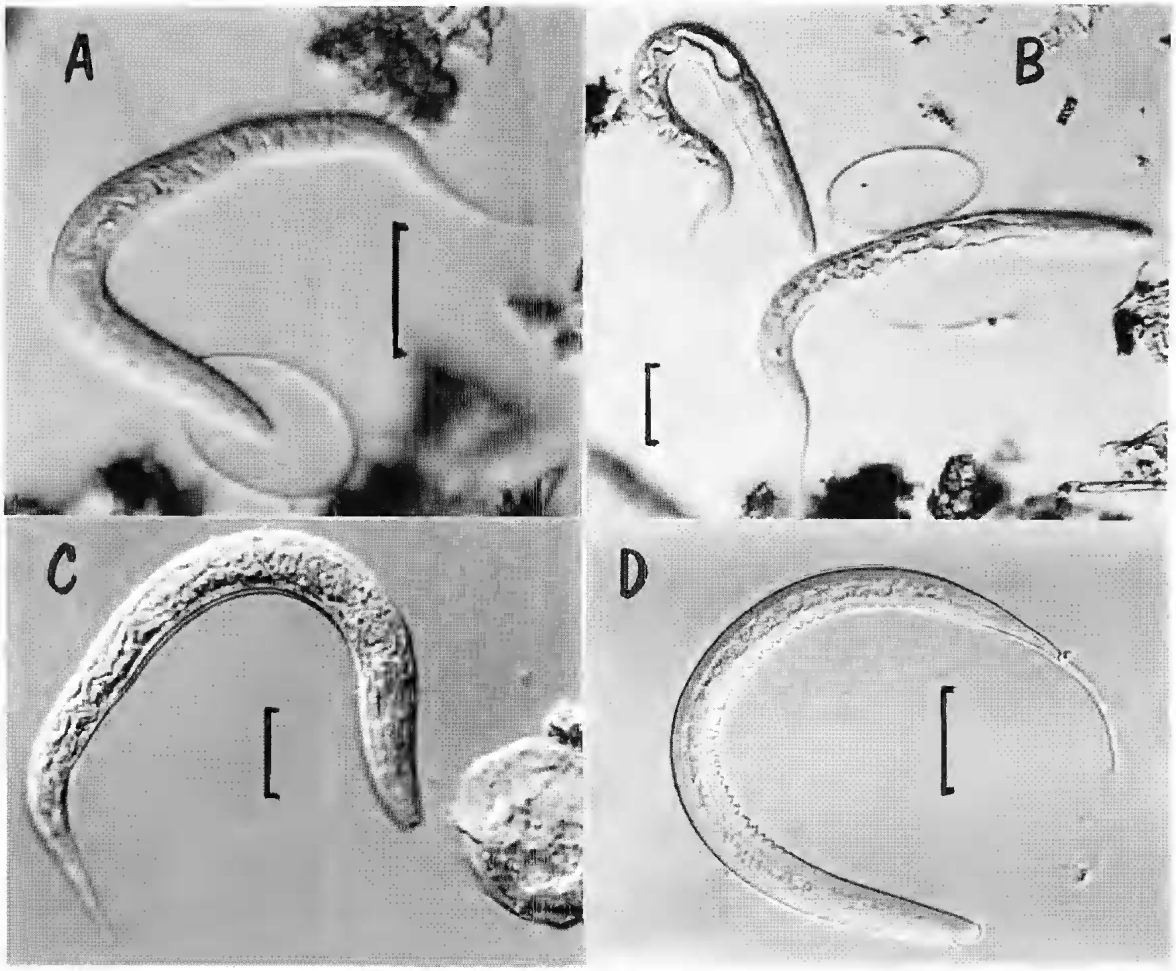

Fig. 3. *Oesophagostomoides eppingensis* larval development when cultured in distilled water at 25° C for 4 days. A. Hatching. B. First-stage larva, day 1. C. Second-stage larva, day 3. D. Third-stage larva, day 4. Scale bars = 40 µm A – C; 70 µm D.

(2000) for the superfamily Strongyloidea, to which *Oesophagostomoides* belongs.

The process of hatching, including increased larval movement, for *O. eppingensis* follows the basic pattern suggested by Bird & Bird (1991) as common to all nematodes. The escape of the larva by mechanical disruption of part of the egg shell is similar to that described for *L. eugenii* (Smales 1977) and could therefore also involve enzyme action to effect a change in permeability of the egg and increase plasticity of the shell (Smales 1977).

Examination of eggs and larvae confirmed previous suggestions (Smales 1994; Gerhardt *et al.* 2000) that only two species of intestinal nematode occur in *L. krefftii*. Measurements of eggs in this study (88 – 92 µm x 42 – 50 µm) are consistent with measurements of eggs of *O. eppingensis* and fall within the size range of eggs of other intestinal nematodes occurring in wombats (Beveridge 1978). The range of temperatures at which egg hatching occurred, 18 – 30° C is consistent with that recorded for other strongylids. For example, the eggs of *Chabertia ovina* (Fabricius, 1788) hatch between 6 and 36° C, *Strongylus vulgaris* (Looss, 1900) between 8 and 39° C, *Oesophagostomum columbianum* Curtice, 1890 between 15 and 37° C and *Castorstrongylus castoris* Chopin, 1925 between 18 and 25° C (Anderson 2000). Given the hot, dry climate of EFNP, we expected that the optimum temperature for hatching would have been at the high end of the range such as, for example, 30° C recorded for *S. vulgaris* and *O. columbianum* (Anderson 2000). The optimum was, however, 26° C, a temperature reported as optimum for *C. ovina* and close to the 25° C optimum reported for a number of strongylids, such as *Ostertagia ostertagi* (Stiles, 1892), *Trichostrongylus axei*

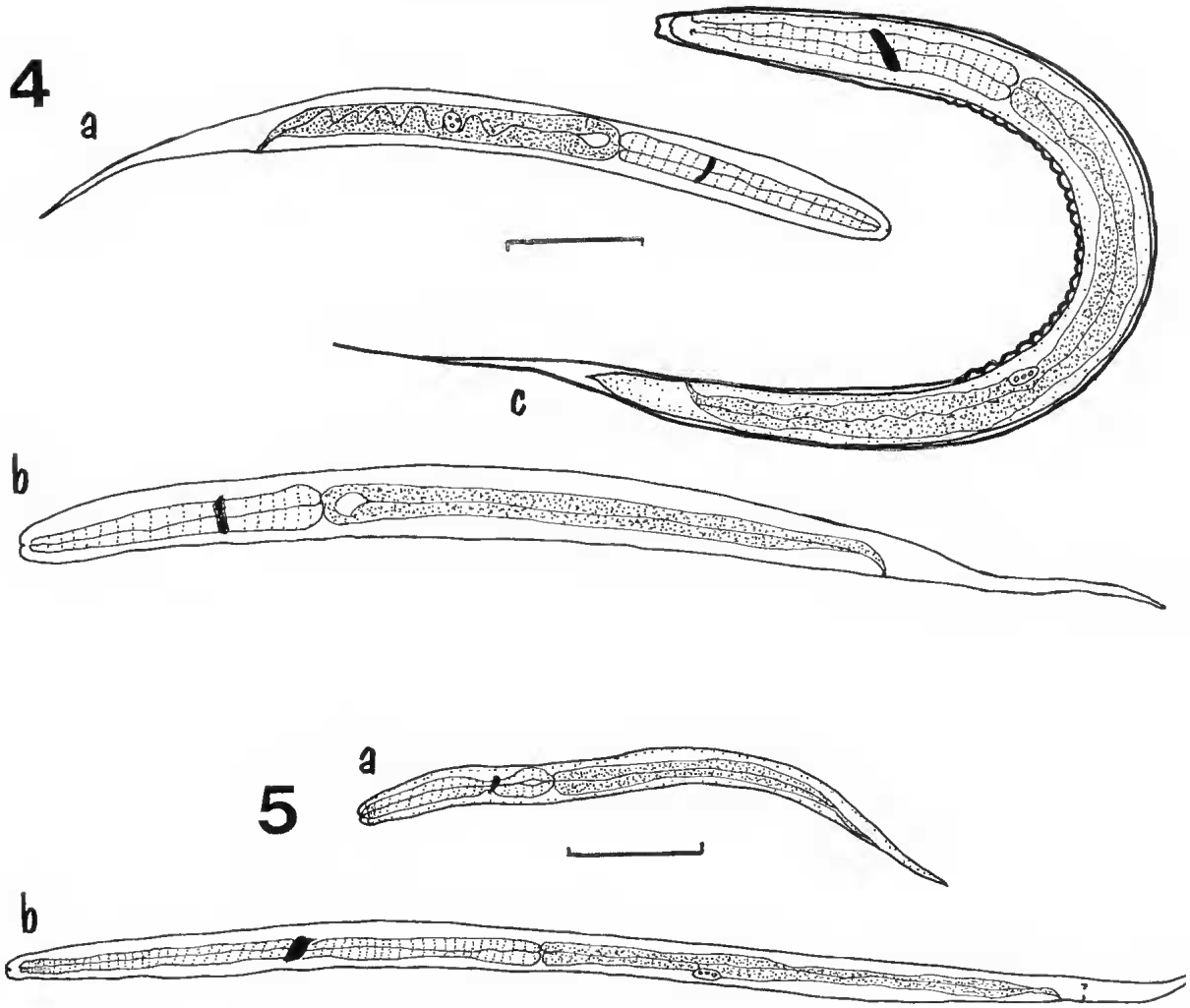

Fig. 4. *Oesophagostomoides eppingensis* free-living stages. a. First-stage larva. b. Second-stage larva. c. Third-stage infective larva. Scale bar = 50 µm.

Fig. 5. *Strongyloides* sp. Free living stages. a. Rhabditiform first or second-stage larva. b. Filariform third-stage infective larva. Scale bar = 50 µm.

(Cobbold 1879), *Syngamous trachea* (Montagu, 1811) and *Ancylostoma caninum* (Ercolani, 1859), found in temperate climates (see Anderson 2000). One possible explanation is that eggs might go into a state of arrested development at high temperatures, as has been reported for the eggs of animal parasitic and plant parasitic nematodes (Waller & Donald 1972; Bird & Bird 1991), to ensure survival.

Of the three morphotypes of larvae found in faecal cultures, the first morphotype was a typical strongylid. No obvious differences in size, growth rate or morphology of this type were detected during culture, lending weight to the presumption that these larvae represented a single species, namely *O. eppingensis*.

The other two morphotypes could clearly be designated developmental larval stages of species typical of the family Strongyloididae. Although both genera *Parastrongyloides* and *Strongyloides* occur in marsupials, only a *Strongyloides* species has been reported from vombatids (Skerrat 1995). Consequently these larvae are presumed to be *Strongyloides* sp., possibly *S. spearei* Skerrat, 1995, occurring in the common wombat (see Skerrat 1995). The infective larvae we found, however, were smaller (mean lengths of 445 µm compared

with 529 µm) with a shorter oesophagus (210 µm compared with 236 µm) a shorter tail (47 µm compared with 79 µm) and with the genital anlage closer to the anterior end (277 µm compared with 324 µm) than in *S. spearei*. They may, therefore, be either a distinct species or represent a population of smaller worms than populations of *S. spearei* from the common wombat. A more detailed examination of all stages of the life cycle, particularly by culturing larvae through to adults, is needed before the specific status of the *Strongyloides* sp. from the northern hairy-nosed wombat can be determined.

The hatching of *L. eugenii* as a sheathed second-stage larva was thought by Smales (1977) to be a protective response to the potential for desiccation of eggs and larvae under Australian climatic conditions. Neither this strategy nor a preference for higher temperatures for hatching success has evolved in *O. eppingensis*. Monitoring for the presence of intestinal helminths in the EFNP population through 1996 (Gerhardt *et al.* 2000) has shown that *O. eppingensis* is present throughout the year. Larvae must, nevertheless, be sufficiently robust to survive the hot summers and dry winters typical of Central Queensland. Further work is needed to determine the level of heat tolerance and responses to desiccation of *O. eppingensis* and how they relate to the life-cycle strategies of the parasite. A better understanding of the dynamics of the free-living stages could be useful when developing management strategies for the wombat host population.

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